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Biochemistry

Methods for the large-scale synthesis of psoralen furan-side monoadducts and diadducts

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ABSTRACT **We report methods for the preparation of multimicromole quantities of extremely pure uniquely photoadducted psoralen–DNA furan-side monoadducts and diadducts (cross-links). The methods use high-intensity krypton and argon ion lasers in the photoreactions and HPLC methods to purify the required oligonucleotides containing the photoadducts. With these methods we have synthesized 2–3 μmol of 8-mer psoralen furan-side monoadduct and diadduct. These methods allow one to generate large amounts of psoralenated DNA oligonucleotides and facilitate their study by NMR and x-ray crystallography.**

Psoralens are linear furocoumarins that have been used for the treatment of certain types of skin diseases (1, 2), antileukemia therapy (photophoresis), and the sterilization of blood products (3, 4). Psoralens photochemically alkylate DNA by reacting with pyrimidines (5). Fig. 1 shows the structure of the three main adducts between thymidine and HMT (5, 6). Reactions take place at the 3,4 or 4',5' double bonds of the psoralen with the 5,6 double bond in pyrimidines. The first step is the intercalation of the planar psoralen between base pairs of a double-stranded nucleic acid. Psoralen then reacts with the pyrimidine bases to form a MAf or a Mpy when UV-irradiated at a long wavelength (320–410 nm). By absorbing a second photon, the MAf can photoreact with adjacent pyrimidine bases on the opposite strand of double-stranded DNA (or RNA) to form an interstrand XL. Mpys do not absorb light at wavelengths above 320 nm and cannot be driv n to form interstrand XLs with long-wavelength UV irradiation. All the photochemical adducts can be photoreversed by exposure to 254-nm UV or, for MAfs and XLs, by treatment with base (7, 8). A solution structure derived from NMR data for the 8-mer 5'-d(GGGTACCC)-3' cross-linked with the psoralen 4'-aminomethyl-4,5',8-trimethylpsoralen has been reported (9).

Psoralens have been very useful as probes for nucleic acid structure and function (10, 11). Transcriptional elongation complexes arrested by psoralen adducts at specific sites on a DNA template have been generated (12–15). Psoralen-DNA adducts are substrates for DNA repair enzymes (16). Other types of protein–DNA interactions have also been studied using psoralens as probes (17–21).

The lack of methods for the large-scale synthesis of psoralen monoadducts and diadducts is a major unsolved problem in the research on psoralenated DNA oligonucleotides. Traditional methods used Hg/Xe arc lamps (22) and black-ray light (23) for investigation of the photochemistry of psoralen–DNA interactions. Photochemical efficiency with these light sources is very low and often results in the generation of multiple photoproducts, despite the use of appropriate filters to achieve monochromicity. Traditional methods rely on denaturing PAGE followed by DNA elution from gel slices to separate the phot adducted DNA oligonucleotides from other components in the reaction mixture. This procedure results in considerable loss of DNA during sample work-up and the persistence of contaminating photoproducts and impurities released from gel slices. By using PAGE, an upper limit of a couple hundred micrograms of psoralen-adducted DNA can be prepared at one time. X-ray crystallographic or NMR studies to determine the structures of DNA–psoralen adducts and their complexes with repair enzymes and RNA polymerases require large amounts of extremely pure psoralen-adducted molecules. Here we report synthetic methods that overcome conventional difficulties. We use high-intensity laser light to synthesize unique MAfs and XLs and, subsequently, purify the photoadducts by HPLC. The final products are extremely (>95%) pure and the yields are in multimicromole quantities.

MATERIALS AND METHODS

Materials. HMT (HRI Associates, Concord, CA) stock solutions (≈0.5 M) were prepared in dimethyl sulfoxide. Oligonucleotides were synthesized on an Applied Biosystems automated DNA synthesizer. Argon ion and krypton ion lasers were purchased from Spectra-Physics. Quartz flow cells were from NSG Precision Cells (Hicksville, NY). HPLC instrumentation was purchased from Beckman and Rainin Instruments. All HPLC was performed at room temperature, and no particular effort was made to regulate the temperature of the columns during the chromatography.

Oligonucleotide Work-Up. Oligomers were deblocked with ammonia and purified by reverse-phase HPLC with the trityl group on (Rainin Instruments catalogue).

Preparation of DNA–HMT MAfs. *Krypton ion laser irradiation.* Irradiations were carried out using a Spectra-Physics 2020 krypton laser operating in broad-band mode at 406.7 nm and 413 nm at 350 mW. Oligonucleotides were dissolved in 32 ml of 150 mM NaCl/10 mM $MgCl_2$/1 mM EDTA/15 mM azide/0.16 mM HMT. A quartz cuvette (10-cm path length) containing this solution was placed in the laser beam between two dielectric mirrors optimized for reflectivity at 406.7 nm with stirring (Fig. 2*A*). The mirrors caused the laser beam to reflect a total of eight passes through the sample. During the irradiation, 5 μl of the DNA solution was withdrawn from the cuvette for gel analysis. After the irradiation, the DNA was recovered by EtOH precipitation (24).

HPLC analysis. The DNA was dissolved in 1.0 ml of 9.5% (vol/vol) acetonitrile/100 mM triethylammonium acetate, pH 6.5, and applied to a Dynamax 4.6 mm × 25 cm reverse-phase C_{18} column. The DNA was eluted with a linear acetonitril gradient in 100 mM triethylammonium acetat (pH 6.5) over 80 min (flow rate = 1.0 ml/min). The percentage of acetonitrile was changed from 9.5 to 17.5% from 5 to 85 min. The fractions of interest were lyophilized and resus-

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Abbreviations: HMT, 4'-hydroxymethyl-4,5',8-trimethylpsoralen; UM, unmodified; XL, cross-link; MAf, furan-side monoadduct; Mpy, pyrone-side monoadduct.

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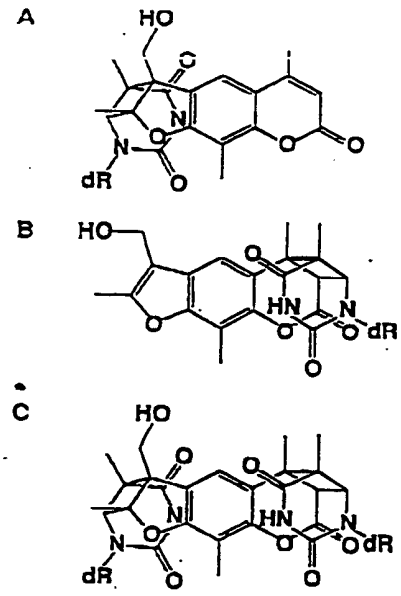

FIG. 1. Structures of the three main types of 4′-hydroxymethyl-4,5′,8-trimethylpsoralen (HMT)-thymidine adducts formed during the photoreaction of HMT with DNA. (*A*) Furan-side monoadduct (MAf), *cis–syn* conformation. (*B*) Pyrone-side monoadduct (Mpy), *cis–syn* conformation. (*C*) Diadduct (cross-link, XL), *cis–syn* conformation.

pended in a minimum volume of TE (10 mM Tris·HCl, pH 7.5/1 mM EDTA), and the DNA was recovered by EtOH precipitation.

Preparation of DNA–Psoralen XLs. *Annealing of the DNA strands.* Self-complementary 8-mer DNA (10–50 μmol) was dissolved in 0.2–1.0 liter of annealing buffer (50 mM Tris·Cl, pH 7.5/10 mM $MgCl_2$/100 mM NaCl/1 mM EDTA/15 mM sodium azide/0.16 mM HMT). Annealing was done by heating the DNA solution to 95°C for 30–40 min and slow cooling to room temperature overnight.

Argon ion laser irradiation. Irradiations at room temperature (22–23°C) were carried out with a Spectra-Physics 2045 argon laser operating in broad-band mode centered at 366 nm. The HMT/DNA solution was pumped through a jacketed quartz flow cell (10-mm path length) that was positioned in the path of the laser light (Fig. 2*B*). The power output of the laser was ≈5 W. A cylindrical quartz lens was used to focus the light beam to illuminate the entire area of the flow cell. The surface intensity of light in the cell was 30–37 W/cm². The DNA solution was pumped through the flow cell at 5–10 ml/min with a peristaltic pump. To achieve maximum yield

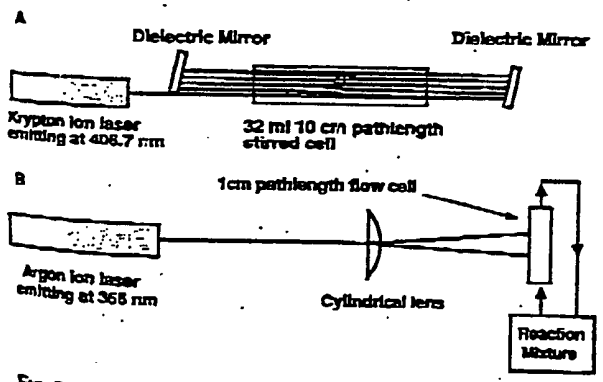

FIG. 2. Schematic diagrams for the optical path during sample irradiation using the krypton laser (*A*) and the argon laser (*B*).

of XL, the DNA solution was irradiated for two cycles with one addition of a fresh dose of 0.16 mM HMT in between cycles. After irradiation, the reaction mixture was lyophilized to ≈50 ml, dialyzed against 500–1000 vol of water, and lyophilized.

HPLC purification. Both reverse-phase chromatography and anion-exchange chromatography were used for the purification of the 8-mer XL (8XL) DNA. See the preparation of the MAf for a general description of the reverse-phas purification method. The specific variations are given in Fig. 4. Ion-exchange HPLC was on a Nucleogen (Machery-Nagel) DEAE column. The DNA samples were injected in ≈1.5 ml of 30% acetonitrile/20 mM sodium acetate, pH 5.5, and eluted with a linear KCl gradient over 200 min (flow rate = 1.0 ml/min). The KCl gradient was changed from 0.0 to 1.0 M from 5 to 205 min. Fractions enriched for cross-linked DNA were then dialyzed extensively against water, lyophilized to dryness, and resuspended in a minimum volume of TE, and the DNA was recovered by EtOH precipitation.

Gel Analysis of HPLC Fractions. DNA from 1 μl of the pertinent HPLC fractions was 5′-^{32}P-end-labeled using T4 polynucleotide kinase and [γ-^{32}P]ATP and visualized by autoradiography (24). Relative amounts of monoadduct or XL were determined by excising the gel slices and measuring the ^{32}P radioactivity by scintillation counting.

RESULTS

Strategy for the Large-Scale Synthesis of MAf. A comparison of the UV absorption spectra of DNA, MAf, and psoralen reveals that DNA stops absorbing light at $\lambda > 310$ nm whereas the absorption cutoff for psoralen MAf DNA extends to >395 nm. Psoralen continues to absorb light to at least 410 nm (see refs. 5 and 25). The absorption coefficient of psoralen at wavelengths longer than 400 nm is only between 1 and 20 ($1\ mol^{-1}\cdot cm^{-1}$). To effect efficient photochemistry at these wavelengths, a high-intensity monochromatic light source is necessary. A krypton ion laser emitting light at 406.7 nm and 413 nm was our light source. The laser radiation specifically excites the psoralen to form MAfs with DNA. The resulting monoadducts do not absorb light at the irradiation wavelengths allowing the adducts to accumulate in the reaction. A laser is desirable for this procedure because of its high-intensity collimated light, low-beam divergence, and, most importantly, the monochromaticity of the beam. Because psoralen has such a low extinction coefficient at the wavelength of the krypton ion laser, a long-path-length reaction cell with a multipass irradiation set-up was used to maximize the use of photons (Fig. 2*A*). Two dielectric mirrors reflect the beam of light eight times through the cell. The DNA solution is constantly stirred during the irradiation to achieve uniform exposure of the DNA/HMT mixture. By using a self-complementary DNA 8-mer (5′-GCGTACGC-3′) and HMT, a yield of 30% 8-mer MAf (8 MAf) was achieved after HPLC purification.

Purification of MAf. A self-complementary DNA 8-mer (5′-GCGTACGC-3′) was chosen to illustrate our procedure to generate large quantities of purified MAfs. Other sequences of different lengths have also been monoadducted and their purification is similar (data not shown). Primary psoralen photoadduction occurs at the 5′-TpA-3′ site in the 8-mer (26). Fig. 3 shows a typical reversed-phase HPLC profile of 8-mer/HMT irradiated with (406.7 nm and 413 nm) krypton laser light. The first large slightly asymmetric peak area about 20 min is unmodified 8-mer (8UM) DNA and the second symmetric slightly smaller peak at 30 min is the 8MAf. The contents of these fractions were verified by gel analysis of ^{32}P-labeled DNA (data not shown). Quantification of the respective peak fractions revealed that ≈30% of the total starting DNA was converted to the 8MAf. The second peak

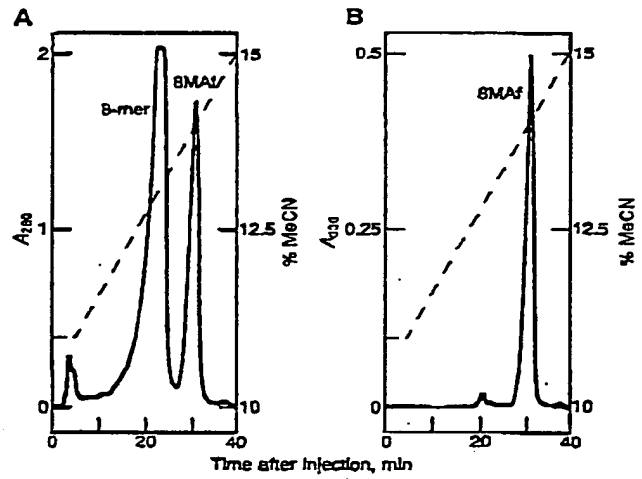

FIG. 3. C_{18} HPLC profiles of the purification of 8MAf. Because of the large amounts of DNA loaded on the C_{18} column, we monitored the absorbance at 330 nm instead of at 260 nm so that the peaks would stay within the scale (<2) of sensitivity of our spectrophotometer.

fractions (fractions 28–31) were pooled and reinjected on HPLC for a second round of purification. 8MAf eluted as a single sharp peak in the second round of C_{18} purification (Fig. 3*B*). Based on autoradiography of the radiolabeled DNA from the large peak in Fig. 3*B*, the purity of the 8MAf was estimated to be >95%. By repeating the above procedure on several batches of 8-mer DNA preparations, we accumulated 3 μmol of 8MAf within a period of a few weeks. A ^{32}P-labeled 8MAf sample of the large-scale preparation [see Fig. 5 (lane 2)]. The DNA migrates as a single band, free of 8UM or other types of psoralen adducts, thus attesting to the purity of the final product. Table 1 gives the sequences and yields of various DNA oligomers monoadducted with HMT that were purified to homogeneity using the above procedure. These sequences were selected for monoaddition because they contained a minimum number of thymidines and for their utility in other projects in our laboratory. Complementary sequences were mixed together before the samples were irradiated, because the psoralen–DNA photoreaction is double-stranded-dependent. Some care must be exercised when choosing complementary sequences to avoid complications during synthesis and purification. We used complementary strands that were of different lengths and contained a 5′-TpA-3′ site in the double-stranded region. Each DNA oligomer of different length had a different characteristic HPLC retention time. The total yields of monoadducted oligonucleotides varied in an unpredictable manner with the DNA sequence. To purify monoadducted oligomers longer than 25 nucleotides, we resorted to preparative denaturing PAGE (30MAf in Table 1). This procedure is much more time and labor intensive and severely limits the quantities of material that can be conveniently prepared.

Strategy for the Large-Scale Synthesis of 8-Mer Diadduct. Production of preparative quantities of cross-linked HMT-DNA was investigated. An argon ion laser was our high-intensity light source (366 nm). Both HMT and th MAfs absorb light efficiently at 366 nm. The quantum yield for the conversion of MAf t XL is 4 times greater than the quantum yield for formation of monoadduct from HMT. Therefore, the MAf does not accumulat in the reaction mixture. The DNA/HMT mixture is pumped through a beam of argon laser light (Fig. 2*B*). The absorbance of a saturated solution of HMT at this wavelength of irradiation is 0.36 *A* units for a 1-cm path length. Under these conditions, the entire sample

Table 1. MAfs synthesized

Length, nt	MAf oligomer sequence	Quantity	% yield
8	5′-GCGTACGC-3′	3.0 μmol	30.0
8*	5′-GGGTACCG-3′	14.0 nmol	6.0
13*	5′-GCTCGGTACCCG-G-3′	7.0 nmol	3.0
8†	5′-TCGTAGCT-3′	0.5 μmol	10.0
12†	5′-GAAGCTACGAGC-3′	0.7 μmol	14.0
19	5′-GATCCCCGGGTACCGAGCT-3′	21.0 nmol	16.0
21	5′-CGGAATTCCGGGTACCGGCCG-3′	90.0 nmol	19.4
24	5′-GATCGCTCCCGGGTACCGAGCTCG-3′	26.0 nmol	4.1
25	5′-GATCCGGCCGGGTACCCGGAATTCCG-3′	10.0 nmol	9.3
30	5′-TTTGCCGATCCCCCCCCTTGCCATAGACCGA-3′	20.7 nmol	20.0

nt, Nucleotide.
*These two oligonucleotides are partially complementary to each other.
†These two oligonucleotides are partially complementary to each other.

is exposed to an adequate amount of light for conversion to XL. A couple of cycles of exposure to laser light ensures maximum conversion of the 8-mer DNA to XL. The rate of pumping can be as fast as the peristaltic pump would allow, normally ≈10 ml/min thus shortening the irradiation time and the operating expense of the laser. The high intensity of the laser light source permitted the conversion of large quantities of DNA into XL in a reasonable amount of time (1–2 hr). We have prepared 3 μmol of highly purified 8-mer XL (8XL) formed between the DNA octanucleotide (5′-GCGTACGC-3′) and HMT with a 36–80% total yield. The greatest yield was obtained with HPLC-purified 8-mer, and the lowest yield was obtained with a crude 8-mer preparation.

Purification of Diadduct. High levels of photoaddnction of HMT to 8-mer (5′-GCGTACGC-3′) using argon laser resulted in multiple psoralen additions. Purification of 8-mer specifically cross-linked at the 5′-TpA-3′ site included a combination of reversed-phase and anion-exchange HPLC. After the laser irradiation, the first step was a large-scale C_{18} reverse-phase chromatography. The laser-irradiated DNA separated into two broad asymmetric peaks in the first round of C_{18} HPLC (Fig. 4*A*). For maximum resolution and speed of separation 3–4 μmol of the modified DNA were loaded onto a 25.4 × 250 mm column with each injection. Denaturing electrophoresis and autoradiography (autoradiograph in Fig. 4*A*, fractions 21–34) revealed the presence of predominantly three DNA species. The first peak area (fractions 21–26) contained mostly 8UM and very little of 8XL and 8-mer pyrone-side monoadduct (8Mpy). The second peak area (fractions 29–34) represented mostly 8XL and relatively smaller amounts of Mpy and 8UM. In addition to 8XL some of these fractions contained minute amounts of DNA molecules migrating much slower than 8XL on these gels (data not shown). These faint bands are thought to result from psoralen photoreaction at additional sites other than the preferred single 5′-TpA-3′ site in the 8-mer sequence. These additional bands were eliminated in the subsequent purification steps (see below). Fractions in the second peak area were pooled and subjected to a second round of C_{18} chromatography. The elution profile (Fig. 4*B*) showed that the 8UM was effectively separated from the enriched XL plus 8Mpy material (fractions 33–42 in the gel, Fig. 4). Since fractions 33–42 that have been enriched for 8XL also contained significant amounts of 8Mpy, we used anion-exchange chromatography under denaturing conditions to separate 8XL from 8Mpy. Reverse-phase HPLC is sensitive to the net hydrophobicity of the adducted DNA, which in this case is the same for the singly adducted 8XL and 8Mpy. Hence , a third round of C_{18} HPLC would not be expected to separate 8XL from 8Mpy. Anion-exchange HPLC using a Nucleogen DEAE column under denaturing conditions (30% acetonitrile) separates DNA molecules based on their net negative charge. Double-

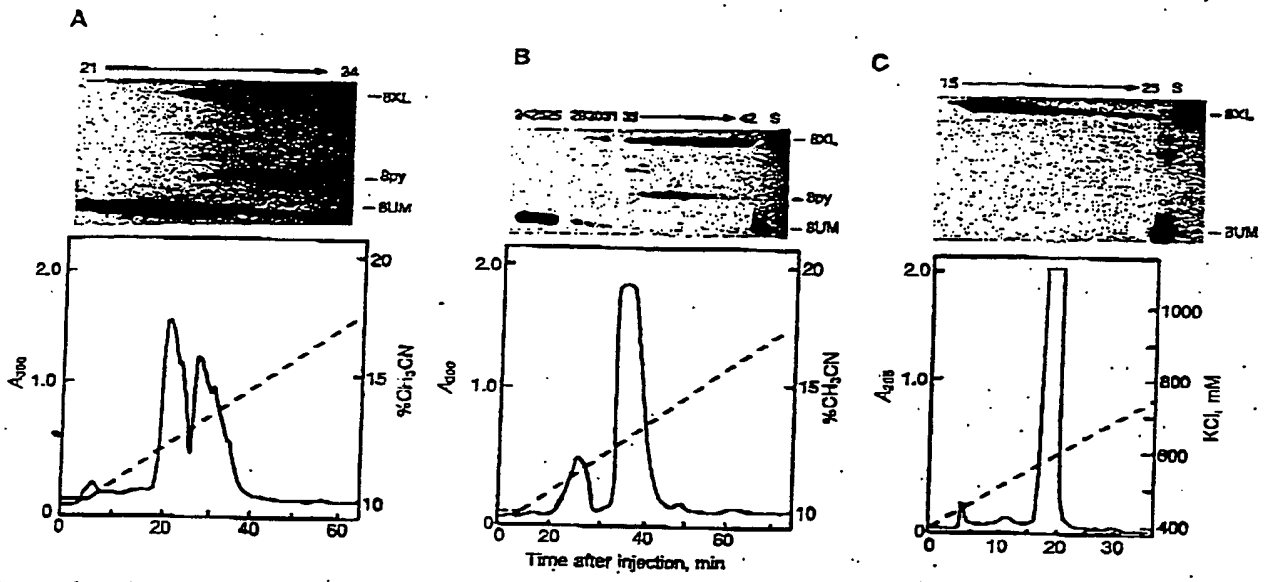

FIG. 4. (*A* and *B*) C_{18} HPLC purification of an 8-mer/HMT mixture irradiated with argon laser light. A Dynamax 60-Å C_{18} column of 2.54 × 25 cm was used in both rounds of purification. The flow rate was 8 ml per min per fraction. From 0 to 10 min, a 10% acetonitrile gradient was used. Then the gradient was changed from 10 to 20% over a period of 80 min. (*C*) DEAE chromatography after two rounds of C_{18} HPLC. The column was a Nucleogen 60-7 DEAE 60 Å with dimensions of 4 × 125 mm. Buffer A is 0.02 M sodium acetate, pH 5.5/30% acetonitrile. Buffer B is 0.02 M sodium acetate, pH 5.5/30% acetonitrile/1 M KCl. The KCl gradient was started at 400 mM at the time of sample injection. The gradient was changed at the rate of 5 mM/min. The flow rate was 1 ml per min per fraction. The upper portion of the HPLC profiles shows the autoradiographic analysis of samples of the relevant HPLC fractions in denaturing polyacrylamide gels. Lanes S contain purified standards of 8UM and 8XL that served as markers. Because of the large amounts of DNA loaded on the C_{18} and DEAE columns, we monitored the absorbance at 300 nm or at 285 nm instead of at 260 nm, so that the peaks would stay within the scale of reasonable sensitivity (<2) of our spectrophotometer.

stranded 8XL displays a greater net negative charge (due to the DNA phosphates) than single-stranded 8Mpy under these conditions. Results of anion-exchange chromatography of the pooled fractions from a second round of C_{18} HPLC showed that 8XL eluted as a single large peak with high purity (see gel in Fig. 4*C*). 8Mpy and the remaining 8UM eluted as a small peak (fractions 10–14, Fig. 4*C*) away from 8XL. Fractions 16–22 in the peak area were pooled and the DNA was recovered by lyophilization. The entire procedure was repeated several times to accumulate multimicromole amounts of 8XL. The Mpy was identified by its absorption spectrum and photochemical behavior (data not shown). It contained ≈5% of the total HMT modified oligomer. We were able to synthesize 3 μmol of >95% pure 8XL (Fig. 5, lane 3) in a few weeks.

Desalting Procedures After HPLC. 8MAf and 8XL eluted from HPLC columns contained triethylammonium and KCl for C_{18} and DEAE chromatography, respectively. Triethylammonium salt was removed by repeatedly (four times) lyophilizing and dissolving the DNA in 60–100% EtOH after each lyophilization. KCl was removed by microdialysis against water. The final desalting step was done on a Sep-Pak C_{18} cartridge (procedure from Waters Associates).

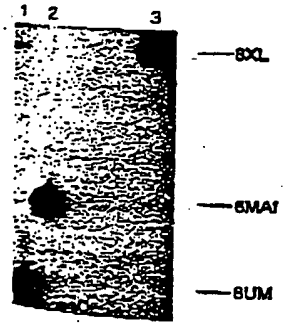

FIG. 5. Autoradiogram of a 24% denaturing polyacrylamide gel showing the typical purity of the final HMT-8-mer photoproducts after HPLC purification. All the DNA samples shown on the gel were 5′-end-labeled with ^{32}P.

DISCUSSION

Site-specific psoralenated DNAs are useful in many biochemical assays. These include substrates for the characterization of DNA repair enzymes (27, 28), elongation complexes of RNA polymerases (12–15), screening of complex genomes (29), induced mutagenesis (30), and DNA structure and dynamics (8–18). There is a clear need to solve the molecular structure of monoadducted and cross-linked DNA molecules by NMR or x-ray crystallography. To this end, these preparative purification methods allow one to synthesize extremely pure monoadducts and XLs.

HPLC purification allows for several times greater loading capacity, a higher degree and speed of resolution, and smaller loss of valuable material than PAGE. Like gel electrophoresis, HPLC methods place an upper limit on the length of a psoralenated DNA oligomer that can be separated from unmodified DNAs. In our hands, adducted oligomers >25 nucleotides were very difficult to purify on C_{18} columns. The increased length of the oligonucleotide neutralizes the relative hydrophobicity differences between the psoralenated molecules and their parent unmodified molecules. Nucleotide sequence of the DNA oligonucleotide also plays an important role in the elution pattern of the adducted oligonucleotide during a reverse-phase separation. For example, monoadducted 10-mers of the sequences 5′-TCCGGGTACC-3′, 5′-GGTACCCGGA-3′, and 5′-GCTCGGTACC-3′ have different retention times—9, 11, 18 min, respectively—on reverse-phase columns under the same elution conditions. The best yields are obtained with HPLC-purified oligonucleotides before they are subjected to a monoaddition or cross-linking reaction. To achieve best resolution, the amounts of DNA loaded on the HPLC columns must be well below

(≈50%) the binding capacity of the columns. This means multiple loadings of the samples to accumulate large amounts of the final monoadducts and XLs.

The distribution of MAfs and XLs in a photoreaction will depend on the rate of MAf formation relative to the rate at which the monoadduct goes on to a XL. This rate is directly proportional to the extinction coefficient of HMT and the MAf at the wavelength at which the irradiation takes place, as represented by the following equations: $k_1 = I\sigma_1\phi_1$; $k_2 = I\sigma_2\phi_2$; $\sigma = 2303\varepsilon/NA$; $k_1/k_2 = (\varepsilon_1\phi_1)/(\varepsilon_2\phi_2)$, where k_1 is the rate of monoadduct formation, k_2 is the rate of XL formation from MAf, I is the intensity of the light irradiating the sample, ϕ_1 and ϕ_2 are the quantum yields for MAf and XL formation, respectively, ε_1 and ε_2 are the extinction coefficients for HMT and MAf, respectively, NA is Avogadro's number, and σ_1 and σ_2 are, respectively, the absorption cross-sections of HMT and MAf. σ is directly proportional to the extinction coefficient of the molecule at that wavelength. The quantum yield ϕ_1 for the formation of MAf of 8-methoxypsoralen, by irradiation at 397.5 nm is 0.0065, and the quantum yield ϕ_2 for th conversion of MAf to XL by irradiation at 341 nm is 0.028 (31). The quantum yield for conversion of the HMT MAf to XL has been measured to be 0.024 by irradiation at 334 nm (32). The quantum yields for the formation of XL from MAf are the same for these two psoralens within experimental error. We assume that the quantum yields for formation of mon adducts from the two psoralens will also be similar. The quantum yield for driving a MAf on to XL is 4 times larger than the quantum yield for the initial formation of MAf. This explains why the MAf does not accumulate in the reaction mixture when a HMT/DNA solution is irradiated at 366 nm (argon laser) where both HMT and the MAf have relatively large extinction coefficients. The extinction coefficients for HMT and the HMT MAf at 406.7 nm and 413 nm were estimated from their UV absorption spectra. The extinction coefficient for HMT at this wavelength is between 1 and 20 $(1\ mol)^{-1}cm^{-1}$), and for the MAf, the extinction coefficient is <1 $(1\ mol^{-1}cm^{-1})$. If a mixture of DNA and HMT is irradiated with light in a region of the absorption spectrum where the extinction coefficient for the MAf is substantially lower than the extinction coefficient for the free psoralen (as for the krypton laser) MAf will accumulate in the reaction mixture. The absorption band of HMT at 397.5 nm and 406.7 nm is the same, so that the quantum yield for conversion of HMT to MAf is expected to be the same at these wavelengths.

Although the procedure we employed to convert large quantities of DNA into XL involved the use of a high-intensity continuous wave laser light source, we are convinced that no biphotonic absorbance and subsequent degradative chemistry of the DNA took place. The rate of absorption of a photon is given by $k = I\sigma$. For a continuous wave laser emitting at 366 nm with a power of 5 W over an area of 0.16 cm^2, I is 5.8×10^{19} photons per s per cm^2. The extinction coefficient for psoralen at this wavelength is 2000. In this case $k = 400\ s^{-1}$, or each molecule absorbs a photon on average once every 2.3 ms. The singlet excited-state lifetime for psoralen has been measured to be ≈2 ns (33, 34). It is unlikely that any significant number of excited psoralen molecules could absorb a second ph ton under conditions we used to synthesize XL. Biphotonic absorption must be considered when pulsed lasers with high peak powers are employed but not with typical continuous wave lasers. After laser irradiation, portions of the DNA samples were 5'-end-labeled with ^{32}P and were heat d at 95°C in 8 M urea before electrophoresis on a denaturing polyacrylamide gel. Any degradation of the DNA would be apparent by this analysis. We hav n ver observed any DNA chain scission under these conditions.

We have obtained ^1H NMR data for the 8MAf and 8XL confirming the presence of a single HMT adduct at the 5'-TpA-3' site.

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